J. Clin. Chem. Clin. Biochem.
Vol. 22, 1984, pp. 545–549

High Resolution Isoelectric Focusing in a Narrow pH Interval for the Phenotyping of Vitamin D-Binding Protein (Gc-Globulin)[1])

By *J. Lizana* and *I. Olsson*

The Electrophoresis Group, Pharmacia Fine Chemicals AB, Uppsala, Sweden

(Received January 19/March 29, 1984)

Summary: A routine method for the analysis of Vitamin D-binding protein (Gc-globulin) phenotypes has been developed. The assay is based on isoelectric focusing in a narrow pH interval (4.5–5.4) using Agarose IEF (1%) or polyacrylamide (T5% C3%) gels, followed by immunofixation. When focused in this pH gradient the anodal and cathodal bands of the phenotype Gc 1–2 are separated by 16–18 mm. The "fast" and "slow" bands of the phenotype Gc 1–1 can also be easily distinguished from each other.

The isoelectric focusing technique which has been developed resolves the Vitamin D-binding protein phenotypes in a fast, convenient and reproducible manner. Since agarose gels are so easy to cast and process, Agarose IEF is recommended as the matrix of choice.

Hochauflösende isoelektrische Fokussierung in einem engen pH-Bereich zur Phänotypisierung des Vitamin D bindenden Proteins (Gc-Globulin)

Zusammenfassung: Für die Analyse von Phänotypen des Vitamin D bindenden Proteins (Gc-Globulin) wurde eine Routine-Methode entwickelt. Der Nachweis basiert auf einer isoelektrischen Fokussierung in einem engen pH-Bereich (4,5–5,4) mit Agarose IEF (1%) oder Polyacrylamid (T5% C3%) als Matrix mit angeschlossener Immunfixation. Nach der Fokussierung in diesem pH-Gradienten sind die anodischen und kathodischen Banden des Gc-Typs 1–2 16 bis 18 mm weit voneinander getrennt. Auch die „fast" und „slow" Bande des Gc-Typs 1–1 können leicht voneinander unterschieden werden.

Die entwickelte Methode unterscheidet mit Hilfe der isoelektrischen Fokussierung die Phänotypen des Vitamin D bindenden Proteins auf schnelle, einfache und reproduzierbare Weise. Da Agarose-Gele leicht zu gießen und anzufärben sind, ist Agarose IEF die empfohlene Matrix der Wahl.

Introduction

Vitamin D and its metabolites are transported in serum by a protein known as Vitamin D-binding protein or Group-specific component (Gc-globulin). This carrier protein has been extensively used as a marker for forensic and anthropological studies. Six common phenotypes are determined by the alleles Gc 1F, Gc 1S and Gc 2, and around 40 rare alleles have been described in the various populations studied (1–3). The serum levels of Gc-globulin are not altered in disorders of calcium homeostasis and vitamin D deficiency or excess (4). However, decreased levels of Gc-globulin in liver disease have been reported (5–8). Little attention has been given to qualitative variations of this carrier protein, but it appears that differences in the Gc-globulin phenotype distribution exist between patients with liver disease and healthy subjects (7). An unusual sialylation of the Gc 1 allele in patients with alcoholic cirrhosis of the liver has recently been reported (8). These reports suggest that the analysis of Gc-globulin phenotypes can be of significant value in other yet unexplored clinical situations.

[1]) Presented in part at the 5th European Congress of Clinical Chemistry, Budapest, Hungary, July 1983.

The Gc-globulin phenotypes are currently analysed by isoelectric focusing (IEF) in polyacrylamide (9, 10) or agarose gels (11–13) followed by immunofixation with monospecific antiserum. The six common Gc-globulin phenotypes focus in a very narrow pH interval (4.8–5.1) and resolution by IEF requires the use of long focusing time (over 4 hours) to allow the cathodic drift to flatten the pH gradient (9, 10), or the addition of a chemical spacer (3-morpholinopropane sulphonic acid, MOPS) to the IEF plate (13). Recently, isoelectric focusing in an immobilized pH gradient has been used to separate Gc-globulin phenotypes. The technique requires time-consuming procedures for preparing the polyacrylamide gels and overnight runs. In addition, agarose gels cannot be used (14).

Routine testing of Gc-globulin requires an easily performed, reliable and fast method with sufficient resolution to distinguish between the Gc-1F (fast) and Gc-1S (slow) variants. Using new narrow range carrier ampholytes (Pharmalyte® 4.5–5.4) we have developed a routine method for the analysis of serum Gc-globulin phenotypes in agarose and polyacrylamide gels.

Materials and Methods

Reagents

Carrier ampholytes Pharmalyte® 4.5–5.4, Agarose IEF, Sorbitol and Silane A-174 were from Pharmacia Fine Chemicals, Uppsala, Sweden. Acrylamide, Bis-acrylamide and Coomassie Brilliant Blue R-250 were obtained from Fluka AG, Buchs, Switzerland. Rabbit Anti Gc-globulin was purchased from Dakopatts AS, Copenhagen, Denmark. All other chemicals used were of analytical grade.

Serum samples were obtained from healthy individuals. Some typified sera were kindly supplied by Dr. *K. Nye*, Dept. Forensic Science, London Hospital Medical College, England.

Equipment

Isoelectric focusing was performed on a Flat Bed Apparatus connected to an Electrophoresis Constant Power Supply equipped with a Volthour Integrator (all from Pharmacia). Water at 15 °C was circulated through the cooling plate of the apparatus. pH was measured with an Ingold surface electrode.

Isoelectric focusing in Agarose gels

Agarose IEF gels (225 × 114 × 1 mm) were cast on GelBond® film (Marine Colloids, Maine, USA). The gel formulation is shown in table 1. The gels can be stored in a humidity chamber at 4 °C for up to 2 weeks. Thick electrode strips were used to absorb any moisture expelled during the run. Electrode solutions were 0.1 mol/l NaOH (catholyte) and 0.04 mol/l glutamic acid (anolyte). The Agarose IEF gels were prefocused for 400 volt hours (around 30 min) at 5 W with set voltage and current of 1500 V and 150 mA, respectively. Then the serum samples (1 µl) were applied directly onto the gel surface using a plastic sample applicator mask placed 1.5 cm from the cathode.

Tab. 1. Gel composition for Gc-globulin: Agarose IEF.

Agarose IEF	0.3 g
Sorbitol	3.6 g
Pharmalyte 4.5–5.4	1.9 ml
Distilled water	27.0 ml

Add Agarose IEF and Sorbitol to the water and dissolve by boiling. Cool to 70 °C and add Pharmalyte. Mix and pour in gel casting frame. Gel dimensions 225 × 114 × 1 mm.

Thereafter focusing was continued for another 1600 volt hours (around 90 min) at 15 W, 1500 V, 150 mA. Twenty samples were run in one gel. The plastic sample applicator was removed after 20–30 min of focusing. Immediately after focusing the gel was submitted to immunofixation (see below).

Isoelectric focusing in polyacrylamide gels

Polyacrylamide gels 215 × 105 × 1 mm were cast on silanized glass plates or GelBond®-PAG film using a capillary casting mould (Pharmacia). The formulation of the gels (T5% C3%) is shown in table 2. The gels can be wrapped in plastic foil and stored in the cool room (+4 °C) for several weeks. Electrode solutions were the same as above.

Tab. 2. Gel composition for Gc-globulin: Polyacrylamide.

Stock Acrylamide/Bis-acrylamide (T10% C3%)	15.0 ml
Glycerol	4.0 ml
Pharmalyte 4.5–5.4	1.9 ml
Distilled water to	30.0 ml
Mix & degass the solution, add TEMED	10.0 µl
Ammonium persulphate (22.8 µg/µl)	300.0 µl

Mix by swirling and pour in capillary casting mould. Gel dimensions 215 × 105 × 1 mm.

The gels were prefocused for 500 volt hours (around 30 min) at 10 W with set voltage and current of 3000 V and 150 mA, respectively. Then the serum samples (1 µl) were applied directly onto the gel surface using a plastic sample applicator mask placed 1.5 cm from the cathode. Thereafter focusing was continued for another 3500 volt hours (around 110 min) at 30 W (3000 V, 150 mA). The plastic sample applicator was removed after 20–30 min of focusing. Twenty samples were run in one gel. Immediately after focusing the gel was submitted to immunofixation (see below).

Immunofixation

Immunofixation was carried out with cellulose acetate strips (Sepraphore® III, Gelman Sciences, Inc., Michigan, USA) soaked in rabbit anti Gc-globulin diluted 1:6 with Tris-barbiturate buffer (pH 8.6, I = 0.050) and containing 40 g/l polyethylene glycol (PEG 6000). The addition of PEG 6000 to the buffer enhances the immunoprecipitin reaction and gives an almost instant wetting of the cellulose acetate strip.

The strips were placed for 15 min on the polyacrylamide gels or 5–10 min on the Agarose IEF gels. The strips were then washed in saline (2 × 1 hour), rinsed in distilled water and stained with Coomassie Brilliant Blue R-250 (5 min). After destaining (around 10–15 min) the Gc-phenotype was determined (3).

Results

The shape of the pH gradient and the distribution of the electrical field (V/cm) obtained with Pharmalyte 4.5–5.4 in Agarose IEF and polyacrylamide gels are shown in figures 1 and 2, respectively. The even distribution of the field strength across the gel plate indicates that the gel can withstand high voltages without the appearence of overheated areas, also called "hot spots". This means short running times and increased resolution. The eveness of the field strength distribution also indicates that the focusing capactiy is the same in all parts of the plate. Independent of the matrix used (agarose or polyacrylamide) a distance of 16–18 mm between pH 4.8 and 5.1 was obtained.

The stability of the pH gradient during the experiment is shown in figure 3. pH measurements done in Agarose IEF gels after 1000 and 2000 volt hours of running showed a minimal degree of anodal gradient drift which was less than 0.04 pH units (fig. 3a). In the case of polyacrylamide gels the pH was measured after 2000 and 4000 volt hours of running. The gels showed a cathodic gradient drift which was less than 0.07 pH units (fig. 3b). The observed pH gradient

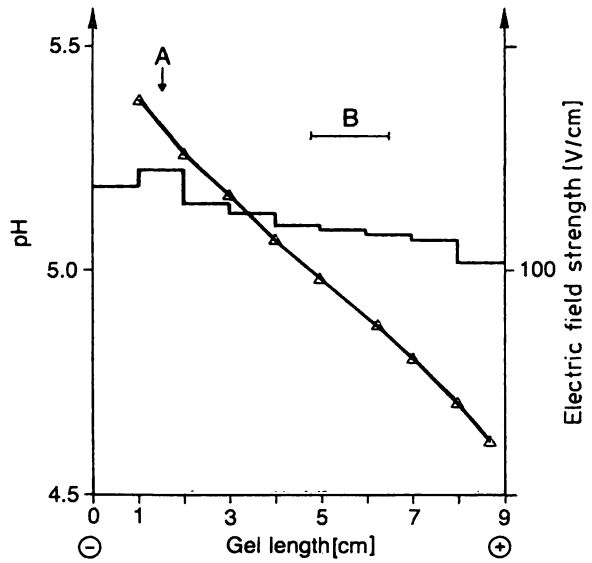

Fig. 1. Electric field distribution and pH gradient (△——△) profile of Pharmalyte 4.5–5.4 in Agarose IEF gels. Measurements were made after 2000 volt hours (power supply settings: 15 W, 1500 V, 150 mA).
A) sample application point.
B) focusing interval of the common Gc-globulin phenotypes.

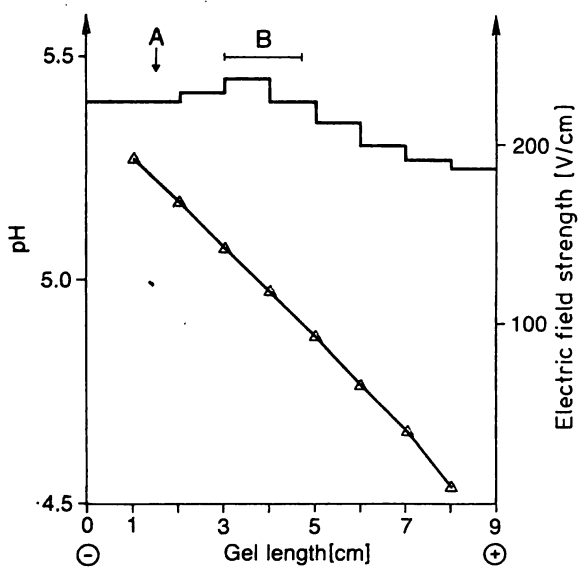

Fig. 2. Electric field distribution and pH gradient (△——△) profile of Pharmalyte 4.5–5.4 in polyacrylamide gels. Measurements were made after 4000 volt hours (power supply settings: 30 W, 3000 V, 150 mA).
A) sample application point.
B) focusing interval of the common Gc-globulin phenotypes.

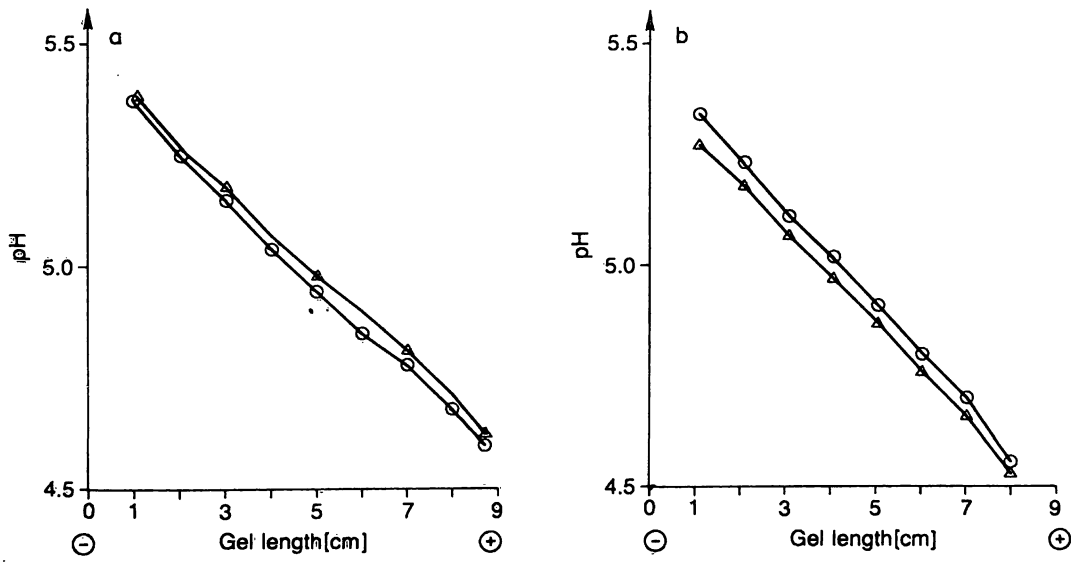

Fig. 3. pH gradient stability of Pharmalyte 4.5–5.4.
a) Agarose IEF gel. The pH gradient was measured after 1000 (O——O) and 2000 (△——△) volt hours (75 and 115 min, respectively).
b) Polyacrylamide gel. The pH gradient was measured after 2000 (O——O) and 4000 (△——△) volt hours (85 and 140 min, respectively).

drift (anodal or cathodal) poses no practical problems when focusing Gc-globulin phenotypes according to the protocol described above.

Immunofixation of Gc-globulin phenotypes after isoelectric focusing in Agarose IEF and polyacrylamide gels is shown in figures 4 and 5, respectively. Slightly better resolution, especially of the phenotype Gc 1F−1S, is obtained with polyacrylamide gels as compared to Agarose IEF gels (compare fig. 4 lane 6 with fig. 5 lane 7). Nevertheless, the resolution obtained with Agarose IEF is quite sufficient for routine use.

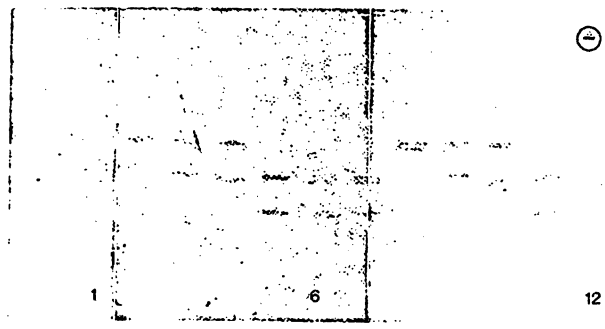

Fig. 4. Immunofixation of Gc-globulin phenotypes after isoelectric focusing in Agarose IEF gels in the pH range 4.5−5.4. Coomassie Brilliant Blue R-250 staining. The phenotypes are as follows:

1. Gc 1S−1F
2. Gc 2−2
3. Gc 1S−2
4. Gc 1F−2
5. Gc 1S−1S
6. Gc 1F−1S
7. Gc 1F−1S
8. Gc 2−2
9. Gc 1S−2
10. Gc 1F−2
11. Gc 1S−1S
12. Gc 1F−1S

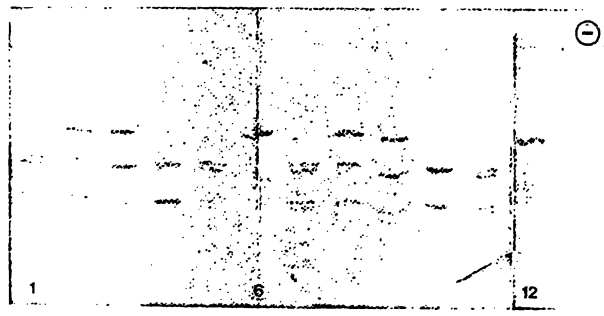

Fig. 5. Immunofixation of Gc-globulin phenotypes after isoelectric focusing in polyacrylamide gels in the pH range 4.5−5.4. Coomasie Brilliant Blue R-250 staining. The phenotypes are as follows:

1. Gc 1F−1S
2. Gc 1S−2
3. Gc 1F−2
4. Gc 1S−1S
5. Gc 1S−1F
6. Gc 2−2
7. Gc 1S−1F
8. Gc 1S−2
9. Gc 1F−2
10. Gc 1S−1S
11. Gc 1F−1S
12. Gc 2−2

Discussion

In this study, a method for the routine testing of Vitamin D-binding protein phenotypes by isoelectric focusing using narrow range carrier ampholytes is presented. Until recently most authors have used polyacrylamide gels (2, 9, 10, 15) but the availability of agarose optimized for use in isoelectric focusing (16) makes this support medium a very attractive possibility, specially in routine testing (11−13). The staff of most clinical laboratories are well acquainted with the handling of agarose gels for zone electrophoresis and immunoelectrophoresis, thus the setting up of agarose isoelectric focusing methods should be easy. The advantages of using agarose gels over polyacrylamide gels are listed in table 3 (17−20). The main problem of the agarose gels has been the pH gradient drift caused by the electroendosmosis and the subsequent flooding of the gel (12, 17). For Agarose IEF this has been largely overcome by the use of a highly purified agarose into which positive charges have been introduced by a chemical reaction (16). The final product is a charge balanced agarose which shows minimum gradient drift under experimental conditions.

Tab. 3. Advantages of using Agarose IEF gels in isoelectric focusing (17−20).

− Fast, simple and reliable gel preparation
− Poses no health hazard to workers
− Chemically unreactive. Does not contain reactive free-radicals or catalysts
− Large molecules can move without restriction in the gel
− Minimal gradient drift
− Very rapid staining and destaining
− Better conditions for the formation of immunoprecipitates
− Transparent dried gel suitable for densitometry and as permanent record
− Familiar gel technique. The staff in routine laboratories are used to handling agarose gels for zone and immunoelectrophoresis.
− Relatively inexpensive

The range, shape and stability of the pH gradient are some of the most important parameters in isoelectric focusing. The carrier ampholyte intervals (pH 4−5, 4−6 or 4−6.5) normally recommended for the study of Gc-globulin are not optimal since Gc-globulin focuses between pH 4.8 and 5.1 (1, 13, 21). Various methods have been used to obtain a suitable pH gradient. *Viau* et al. (15) have exploited the cathodic drift in polyacrylamide gels. This was achieved with

carrier ampholytes pH 4–6 and a long running time, 4.5 hours. Agarose gels have been used by *Thymann* (11) who placed a soft absorbent paper on the gel near the cathodic electrode strip to drain off the excess water produced during the run. A better way to control the flattening of the pH gradient is to use a chemical spacer. This was done by adding MOPS to a mixture of carrier ampholytes pH intervals 4–6.5 and 2.5–5.0 (13). Since charge balanced agarose was used, no flooding of the gel was observed. With this method the running time was shortened to 90 min (2000 volt hours).

The increasing interest in serum Gc-globulin prompted us to develop a fast, simple and reliable method for the routine phenotyping of this carrier protein. The experimental conditions for running isoelectric focusing in both polyacrylamide and Agarose IEF gels are described. In this method a narrow pH interval carrier ampholyte (Pharmalyte 4.5–5.4) is used.

The pH gradient obtained is sufficiently flat to allow a good resolution of the Gc-globulin phenotypes, even of the phenotype Gc 1S–1F, and makes unnecessary the use of chemical spacers or long running times. Since agarose gels are so easy to cast and process and the use of a charge balanced matrix assures a minimum of gradient drift and flooding, we recommend Agarose IEF as the matrix of choice for the routine analysis of serum Gc-globulin phenotypes.

References

1. Putnam, F. W. (1977) In: The plasma proteins (Putnam, F. W., ed.), Vol. 3, Chapter 5, Academic Press, N. Y.
2. Constans, J. & Cleve, H. (1979) Hum. Genet. *48*, 143–149.
3. Cleve, H., Constans, J., Berg, S., Horte, B., Ishimoto, G., Matmasumoto, H., Spees, E. K. & Wever, W. (1981) Hum. Genet. *57*, 312–316.
4. Haddad, I. G. & Walgate, J. (1976) J. Clin. Invest. *58*, 1217–1222.
5. Barragry, J. M., Corless, D., Auton, J. A., Carter, N. D., Long, R. G., Maxwell, J. D. & Switala, S. (1978) Clin. Chim. Acta *87*, 359–365.
6. Imawari, M., Akanuma, Y., Itakura, H., Muto, Y. & De Witt, G. (1979) J. Lab. Clin. Med. *93*, 171–180.
7. Brown, I. R. F., Carter, N. D. & Sood, A. (1979) Clin. Chim. Acta *95*, 75–82.
8. Constans, J., Arlet, Ph., Viau, M. & Bouissou, C. (1983) Clin. Chim. Acta *130*, 219–230.
9. Constans, J., Viau, M., Cleve, H., Jaeger, G., Quilici, J. C. & Palisson, M. J. (1978) Hum. Genet. *41*, 53–60.
10. Kueppers, F. & Harpel, B. (1979) Hum. Hered. *29*, 242–249.
11. Thymann, M. (1980) In: Electrophoresis '79 (Radola, B. J., ed.), pp. 123–128, Walter de Gruyter & Co., Berlin.
12. Thymann, M. (1982) Hum. Hered. *31*, 214–221.
13. Lizana, J., Savill, A. & Olsson, I. (1981) In: Electrophoresis '81, (Allen, R. C. & Arnaud, P., eds.) pp. 549–561, Walter de Gruyter & Co., Berlin.
14. Cleve, H., Patutschnick, W., Postel, W., Weser, J. & Görg, A. (1982) Electrophoresis *3*, 342–345.
15. Viau, M., Constans, J. & Bouissou, C. (1977) Sci. Tools *24*, 25–26.
16. Hansson, H. & Kågedal, L. (1980) 13th FEBS Meeting, Jerusalem, Israel.
17. Vesterberg, O. (1980) In: Electrophoresis '79 (Radola, B. J., ed.), pp. 95–104, Walter de Gruyter & Co., Berlin.
18. Thompson, B. J., Burghes, A. H. M., Dunn, M. J. & Dubowitz, V. (1981) Electrophoresis *2*, 251–258.
19. Lizana, J. & Olsson, I. (1982) In: XI International Congress of Clinical Chemistry (Kaiser, E., Gabl, F., Müller, M. M. & Bayer, M., eds.), pp. 1225–1233, Walter de Gruyter & Co., Berlin.
20. Saravis, C. A. & Zamcheck, N. (1979) J. Immunol. Methods *29*, 91–96.
21. Van Baelen, H., Bouillon, R. & De Moor, P. (1978) J. Biol. Chem. *253*, 6344–6345.

Dr. Jorge Lizana
The Electrophoresis Group
Pharmacia Fine Chemicals AB
Box 175
S-75104 Uppsala